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Failure of Chlorpromazine to Inhibit Fluid Accumulation Caused by *Escherichia coli* Heat-Stable Enterotoxin in Suckling Mice

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We studied the effect of chlorpromazine on fluid accumulation caused by purified heat-stable enterotoxin (ST) from enterotoxigenic *Escherichia coli* in suckling mice. We found that chlorpromazine inactivated ST itself in vitro, but did not inhibit the activity of ST in the intestines.

Chlorpromazine (CPZ) is known to inhibit stimulation of cyclic adenosine-3',5'-monophosphate (cAMP) formation by hormones (13). Thus, Holmgren et al. (4) demonstrated that CPZ inhibits intestinal fluid secretion in mice caused by cholera enterotoxin and *Escherichia coli* heat-labile enterotoxin (LT), which are known to cause intestinal fluid secretion through the formation of intracellular cAMP (2, 9, 10). On the other hand, Abbey and Knoop (1) reported that the secretory activity of *E. coli* heat-stable enterotoxin (ST) was also inhibited by CPZ. Since ST is reported to stimulate guanylate cyclase activity (3, 5), but not adenylate cyclase activity, the question arises of whether CPZ really inhibits the action of ST as reported by Abbey and Knoop (1). During studies on the effect of CPZ on fluid accumulation (FA) induced by ST in suckling mice, we found that CPZ inactivated ST itself in vitro.

MATERIALS AND METHODS

Preparation of ST. ST was isolated and purified from the culture supernatant of *E. coli* 53402 A-1, an ST-producing strain isolated from a human patient, as described previously (12). The purification procedure consisted of protamine sulfate treatment, ultrafiltration through an Amicon PM-10 membrane, diethylaminoethyl-cellulose column chromatography, hydroxyapatite column chromatography, Bio-Gel P-10 gel filtration, 90% ethanol extraction, and preparative polyacrylamide gel disc electrophoresis. The minimum effective dose of the purified ST used in this work was about 2.5 ng in the suckling mouse assay. The minimal amount of ST giving an FA ratio (see below) of more than 0.09 after 4 h was tentatively designated as 1 mouse unit.

Assay of ST in suckling mice. ST activity was assayed in suckling mice as described previously (11). Breeding colonies of white mice (Nippon Clea, Osaka, Japan) were established in our laboratory, and suckling mice of 2 to 3 days old were used in the assays. Doses of 0.1 ml of samples were administered by a gastric tube to mice, with about 0.001% Evans blue as a marker. Four hours after the administration of the sample, animals were killed with chloroform. After confirming the presence of the dye in the intestinal lumen, we removed the entire intestine. The FA ratio of each animal was calculated as the ratio of the weight of the entire intestine to that of the rest of the body.

Other materials. Powdered CPZ hydrochloride from Yoshitomi Pharmaceutical Industry Ltd., Osaka, Japan, was used. It was dissolved in distilled water since it did not dissolve completely in either phosphate-buffered saline (pH 7.0) or phosphate buffer (pH 7.0). In some experiments, commercially available CPZ hydrochloride solution for injection (Contomin; Yoshitomi) was also used. Essentially the same results were obtained with the two preparations.

Purified cholera enterotoxin (7) was kindly provided by N. Ohtomo, Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan.

RESULTS

We examined the effect of CPZ on FA induced by purified ST in suckling mice. When CPZ was administered 30 min before ST administration, significant inhibition of FA by CPZ was observed, whereas no appreciable inhibition was observed when CPZ was administered either simultaneously with ST or 30 min afterwards (Table 1). Although the former result is similar to that of Abbey and Knoop (1), the latter results are inconsistent with their results. Thus, the effect of preincubation of ST with CPZ on FA by ST was examined. When a mixture of ST and CPZ was administered without preincubation (that is, immediately after mixing), no appreciable inhibition of FA by CPZ was observed (Fig. 1). On the other hand, when the mixture was preincubated at 37°C for 30 min, FA by ST was significantly decreased by CPZ, the effect of 2 mouse units of ST being completely prevented by 50 μg of CPZ.

Decrease in the FA ratio was dependent on the duration of the preincubation of ST with CPZ. Even preincubation for only a few minutes

TABLE 1. *Effect of CPZ on FA by ST*

Condition	FA ratio[a]
ST alone[b]	0.122 ± 0.004
CPZ 30 min before ST[c]	0.081 ± 0.009
CPZ and ST simultaneously	0.113 ± 0.016
CPZ 30 min after ST	0.133 ± 0.003

[a] The FA ratio was examined 4 h after ST administration. Values are means ± the standard error of five determinations.
[b] Two mouse units of ST were administered.
[c] Fifty micrograms of CPZ was administered.

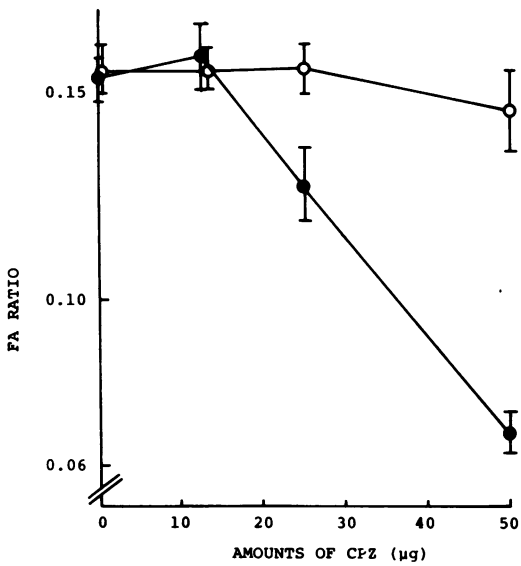

FIG. 1. *Effect of CPZ on FA by ST when administered intragastrically with ST. The indicated amounts of CPZ were mixed with 2 mouse units of ST and administered to mice without incubation (○); or after incubation at 37°C for 30 min (●). The FA ratio with 2 mouse units of ST preincubated at 37°C without CPZ was 0.155 ± 0.005. Values are means ± the standard error of five determinations.*

significantly reduced the ST activity (Table 2). These results suggest that the decrease of the FA ratio on the administration of CPZ with ST was due to the inactivation of ST by CPZ during incubation in vitro rather than to the inhibition of the action of ST by CPZ in vivo.

To test whether CPZ actually inhibits the action of ST on intestinal cells, we examined the effect of intraperitoneal administration of CPZ on ST activity. Intraperitoneal administration of 100 μg of CPZ did not significantly affect ST activity, but it did significantly decrease FA by purified cholera enterotoxin (Table 3). With all the concentrations of ST tested, a slight but appreciable decrease of the FA ratio was observed; however, we found that this might be due to a difference of the response to ST after

TABLE 2. *Effect of preincubation of ST with CPZ on FA by ST*[a]

Preincubation period (min)	FA ratio[b]
0	0.134 ± 0.008
1	0.111 ± 0.013
5	0.089 ± 0.007
10	0.063 ± 0.004
20	0.060 ± 0.002
30	0.062 ± 0.001

[a] Purified ST and CPZ were mixed and preincubated at 37°C for the indicated times. Then doses of 2 mouse units of ST with 50 μg of CPZ were administered to the mice. The FA ratio was examined 4 h after ST administration. The FA ratio with 2 mouse units of ST preincubated at 37°C for 30 min without CPZ was 0.155 ± 0.005.
[b] Values are means ± the standard error of five determinations.

treatment with CPZ. Maximal FA in suckling mice was observed about 3 to 4 h after ST administration (Fig. 2). Thus, all the above observations were made at 4 h after ST administration. On the other hand, when CPZ was administered, maximal FA was observed about 5 to 6 h after ST administration (Fig. 2). The FA ratio of mice 4 h after treatment with ST alone and that of mice 6 h after treatment with both ST and CPZ were quite similar (Table 4). The delay in the response of mice treated with CPZ could be explained by a reduction of intestinal peristaltic movement by CPZ since we found that the movement of the dye from the stomach to the intestine was slowed down by the administration of CPZ.

From these results we conclude that CPZ does not inhibit the action of ST on the intestine and that the observed inhibitory effect of CPZ on ST activity (Fig. 1; Table 2) was due to the inactivation of ST by CPZ.

DISCUSSION

The inhibitory effect of CPZ on intestinal fluid secretion caused by cholera enterotoxin and LT was first observed in mice by Holmgren and his co-workers (4). Later they extended their observations to piglets (6) and human patients (8). In piglets, diarrhea caused by enterotoxigenic *E. coli* was reversed by intramuscular injection of CPZ (6). Rabbani et al. (8) also reported the successful treatment of human cholera by either intramuscular or oral adminstration of CPZ. CPZ has this effect because it inhibits the stimulation of cAMP formation (13), a fact being confirmed by Holmgren et al. (4), who showed that CPZ completely inhibits stimulation of adenylate cyclase in the intestinal mucosal membrane. Lönnroth et al. (6) also demonstrated

TABLE 3. *Effect of CPZ administered intraperitoneally on the action of ST and cholera enterotoxin*[a]

CPZ (μg)	FA ratio				
	ST			Control	Cholera enterotoxin (7 μg)
	2 mouse units	3 mouse units	5 mouse units		
0	0.162 ± 0.007	0.168 ± 0.012	0.188 ± 0.011	0.061 ± 0.002[b]	0.159 ± 0.015
100	0.128 ± 0.009	0.151 ± 0.007	0.169 ± 0.014	0.062 ± 0.002[c]	0.080 ± 0.007

[a] CPZ was administered intraperitoneally, and immediately afterwards the indicated amounts of ST and cholera enterotoxin were administered as described in the text. The FA ratio was examined 4 h after ST administration and 10 h after cholera enterotoxin administration. Values for FA ratios are means ± the standard error of five determinations.

[b] Phosphate-buffered saline (pH 7.0) was administered.

[c] CPZ alone was administered.

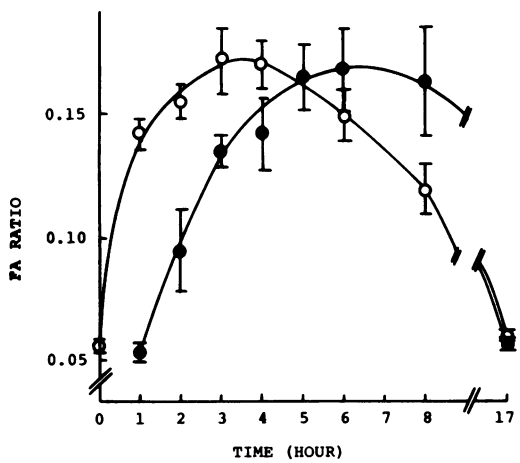

FIG. 2. *Time course of FA caused by ST with or without the intraperitoneal administration of CPZ. Three mouse units of purified ST was administered as described in the text, and FA ratios were examined at the times indicated. Symbols: ○, Without CPZ; ●, 100 μg of CPZ was administered intraperitoneally before ST administration. Values are means ± the standard error of five determinations.*

TABLE 4. *Responses of suckling mice to ST with or without CPZ*[a]

CPZ (μg)	Time of incubation (h)	FA ratio[b]
0	4	0.168 ± 0.012
100	4	0.151 ± 0.007
100	5	0.165 ± 0.014
100	6	0.169 ± 0.016
100	8	0.153 ± 0.021

[a] One hundred micrograms of CPZ was administered intraperitoneally, and immediately afterwards 3 mouse units of purified ST was administered as described in the text. FA ratios were examined at the times indicated.

[b] Values are means ± the standard error of five determinations.

that in piglets the stimulation of intestinal adenylate cyclase activity by enterotoxigenic *E. coli* infection was reduced about 50% by CPZ treatment.

The recent report of Abbey and Knoop (1) that CPZ also inhibits *E. coli* ST prompted us to study the effect of CPZ on ST activity, since it has been reported that ST stimulates guanylate cyclase, but not adenylate cyclase (3). The experimental data reported in this paper are not consistent with those of Abbey and Knoop (1). Although there is a possibility that the conflicting results are in part due to differences in the ST preparations used in the studies of Abbey and Knoop (1) and in those used in ours, we conclude that CPZ inactivates ST itself, but does not inhibit FA caused by ST in vivo.

The in vitro inactivation of ST by CPZ is shown in Fig. 1 and Table 2. Figure 1 confirmed the inhibitory effect of CPZ on FA by ST reported by Abbey and Knoop (1). However, this figure and the data in Table 2 led us to conclude that CPZ inactivates ST. Abbey and Knoop (1) also reported that CPZ inhibits ST activity when administered before or after ST. The results in Fig. 2 show that there was a considerable delay in the response of suckling mice to ST when CPZ was administered. This might be because CPZ reduces peristaltic movement.

Powdered CPZ hydrochloride did not dissolve completely in either phosphate-buffered saline (pH 7.0) or phosphate buffer (pH 7.0), but was dissolved in distilled water. Since ST is unstable at pH 2.0 or less (12), the pH of the CPZ solution in distilled water was measured and confirmed to be 5.01.

Lönnroth et al. (6) reported that CPZ was effective in the treatment of diarrhea in piglets infected with enterotoxigenic *E. coli* producing both LT and ST. If ST were produced in the intestine of infected animals, the data of Lönnroth et al. are not consistent with our conclusion. But these workers reported that no ST could be detected in the small intestinal fluid of the diarrhea-infected piglets, although LT was detected.

Thus, in these piglets, LT, but not ST, was responsible for the diarrhea.

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